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REVIEW

Isomerase and Chaperone Activities of Protein Disulfide Isomerase are Both Required for Its Function as a Foldase

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Abstract—Protein disulfide isomerase (PDI) is not only an isomerase catalyzing the formation of native disulfide bond(s) of nascent peptide, but also a molecular chaperone assisting chain folding. The intrinsic chaperone activity of PDI is independent of its isomerase activity as shown by its ability of promoting *in vitro* reactivation and suppressing aggregation during refolding of denatured proteins containing no disulfide. The -CGHC- active sites of PDI are not required for its chaperone activity and a mutant PDI with no isomerase activity does function *in vitro* and *in vivo*. The peptide binding site of PDI is responsible for its chaperone activity. Both isomerase and chaperone activities are required for PDI to function as a foldase in assisting protein folding, in other words, the foldase activity of PDI consists of both isomerase and chaperone activities.

Key words: protein disulfide isomerase, foldase, protein folding, molecular chaperones

PDI IS BOTH AN ENZYME AND A CHAPERONE

Anfinsen's principle, that the amino acid sequence of a polypeptide contains all the information necessary for its folding to the native structure which is thermodynamically the most stable [1], has been widely accepted. Consequently, it is generally assumed that a denatured peptide chain *in vitro* or a newly synthesized chain *in vivo* is able to fold spontaneously to form its functional conformation with no assistance from other molecules and with no further expenditure of energy. However, a revolutionary concept on protein folding appeared in the last decade in that the folding and assembly of nascent peptides into functional proteins are not always spontaneous but frequently require the assistance of other proteins [2, 3]. This new concept is actually not in contradiction with Anfinsen's principle, but rather extends and deepens our understanding on protein folding in the cell.

Abbreviations: $APLA_2$) acidic phospholipase A_2; GAPDH) D-glyceraldehyde-3-phosphate dehydrogenase; GuHCl) guanidine hydrochloride; ER) endoplasmic reticulum; PDI) protein disulfide isomerase; mPDI) alkylated PDI at active site thiols; PPI) peptidyl prolyl *cis-trans* isomerase.

Recent studies indicate that the accessory proteins can be classified into two groups: molecular chaperones and enzymes [4]. As defined by Ellis [3], molecular chaperones are "a functional class of unrelated families of protein that assist the correct non-covalent assembly of other polypeptide-containing structures *in vivo*, but are not components of these assembled structures when they are performing their normal biological functions". More and more proteins are found to be chaperones and some known proteins have been shown to have chaperone function. The heat-shock proteins form a large group of chaperones. The enzymes, also named foldases, catalyze covalent changes essential for the formation of the native and functional conformations of the proteins concerned. So far only two enzym s, protein disulfide isomerase (PDI), catalyzing the formation of native disulfide bonds [5], and peptidyl prolyl *cis-trans* isomerase (PPI), catalyzing the isomerization of some of the stable *trans* peptidyl prolyl bonds to the *cis* configuration necessary for the functional fold of proteins [6], have been identified to be foldases. The formation of native disulfides and the *cis-trans* isomerization of prolyl imide bonds are both covalent reactions and are frequently rate-limiting steps in the protein folding process. It has therefore been explicitly stated

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by Ellis that PDI is not a chaperone [3]. Later, Freedman, while recognizing that PDI in stoichiometric concentrations increases dramatically the reactivation yield of some denatured proteins, nevertheless, claimed that it is not a general chaperone action [7].

The folding of a peptide chain and the formation of its native disulfide(s) are two processes intimately connected and work in conjunction. It is necessary for the target sequence to fold at least to some extent so as to bring the relevant sulphydryl groups close enough in space for the formation of the correct disulfide bonds. On the other hand, the disulfides once formed will surely affect the subsequent folding and conformational adjustment to form the functional structure. At least under *in vitro* conditions, PDI appears to be able to promote the folding of a peptide chain to a conformation favorable for native disulfide formation without the assistance of chaperone prior to the formation of native disulfide bonds. The spontaneous folding and oxidation of peptides containing sulphydryl groups are often slow processes [8]. Therefore it is logical to ask the question: in addition to the catalysis of the formation of native disulfides, does PDI promote the folding of a peptide to its native conformation as chaperones do?

PDI, a highly unusual multifunctional protein, is present in the endoplasmic reticulum at high concentrations [9] and is remarkably capable of nonspecific peptide binding [10, 11], which are important prerequisites for a protein to be a molecular chaperone. Considering all the properties of PDI, we put forward a hypothesis in 1993 that protein disulfide isomerase is both an enzyme and a chaperone [12]. A similar suggestion has also been made by Noiva [13] and Gilbert [14].

Since then, more and more experimental data, *in vitro* and *in vivo*, have lent support to this hypothesis. Alan Schechter, who worked with Anfinsen at NIH on protein folding, stated in his obituary article for Anfinsen in 1995 that "in fact, Chris and his colleagues—Franco de Lorenzo, David Givol, and Sara Fuchs—were the first to identify and characterize a chaperone, the protein-disulfide isomerase" [15]. In recent years several reviews have covered various aspects of this active area of PDI studies [5, 7, 13, 16-19]. In the present article, I will focus on the chaperone activity of PDI and the relationship with its isomerase activity as a foldase.

THE CHAPERONE ACTIVITY OF PDI IS INDEPENDENT OF ITS ISOMERASE ACTIVITY

PDI assists the *in vitro* folding of proteins containing no disulfide. As peptide chain folding and disulfide bond formation are two intimately connected processes working in cooperation it is hard to distinguish explicitly the possible chaperone activity of PDI from its isomerase activity in assisting the folding of disulfide containing proteins. Therefore we employed proteins containing no disulfide as target proteins to examine whether PDI is indeed capable of assisting the folding of unfolded proteins independent of its disulfide isomerase activity. The homotetrameric D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [20] and monomeric rhodanese [21] were chosen as target proteins for the additional reason that both of these two proteins show low spontaneous reactivation after full denaturation and a remarkable propensity to aggregate in dilute GuHCl.

The presence of PDI in the refolding system at near stoichiometric instead of catalytic amounts indeed increases greatly reactivation yield of both GuHCl-denatured GAPDH and rhodanese upon dilution, and suppresses their aggregation during refolding without being a part of the final functional structure. The reactivation of these two proteins does not involve any covalent change. In addition, PDI suppresses aggregation of rhodanese during thermal denaturation. Therefore the properties of PDI in assisting folding of GAPDH and rhodanese are consistent with the definition by Ellis for chaperones [3], and fully meet the four criteria proposed by Jakob and Buchner [22] for the characterization of a protein as a molecular chaperone: 1) suppression of aggregation during protein folding; 2) suppression of aggregation during protein unfolding; 3) influence on the yield and kinetics of folding; and 4) effects exerted at near stoichiometric levels. As disulfide formation is not involved in the folding of these two enzymes, the effect of PDI in assisting their reactivation has nothing to do with its isomerase activity, but can only be ascribed to its chaperone activity.

In this connection, it is interesting to note that a disulfide bond forming enzyme from the hyperthermophilic archaebacterium *Sulfolobus solfataricus* has been reported to behave like a molecular chaperone in assisting the folding of alcohol and glutamate dehydrogenases from the same organism. Both dehydrogenases contain no disulfide bond [23].

However, no chaperone-like effect of PDI was reported by Lilie et al. on the refolding of the antibody Fab with intact disulfide bonds under oxidizing conditions [24].

The -CGHC- active sites are not required for the chaperone activity of PDI. The two thioredoxin-like -CGHC- sequences of PDI have been characterized to be the active sites for its isomerase activity [25, 26]. It has been shown by Noiva et al. [27] that a single peptide binding site of PDI is located within the region of 451-476 and the cysteines in the -CGHC- active sites are not required for the peptide binding activity. We have

compared directly the effects of alkylation of PDI active site thiols on its isomerase and chaperone activities [28] and found that only the isomerase but not the chaperone activity of the enzyme is abolished, suggesting that the chaperone activity f PDI is independent of its -CGHC- active sites. Th alkylated PDI (mPDI) shows nearly the same ability for increasing reactivation and decreasing aggregation during refolding of denatured GAPDH as that of native PDI [28].

Essential function of mutant PDI with no isomerase activity. Euglp, a homolog of PDI in yeast but with -CLHS- and -CIHS- instead of the -CGHC- active site sequence, is capable of peptide binding but devoid of disulfide isomerase activity. Its over-expression allows yeast cells to grow in the absence of the essential *pdil* gene product. In this case, significant levels of carboxypeptidase Y accumulated in the endoplasmic reticulum (ER) and failed to be transported further, suggesting that Euglp is able to bind and perhaps stabilize newly synthesized proteins in the folding process. In addition, the regulation of Euglp gene in response to the accumulation of proteins in the ER also suggests its possible role in the folding of nascent peptides similar to the chaperone Bip [29]. In this connection LaMantia and Lennartz [30] designed a mutant yeast PDI with -CLHS- and -CIHS- instead of the two active site -CGHC- sequences and only 5% of the wild-type isomerase activity, and found that yeast cells carrying the mutant PDI are still viable, but with a delay in disulfide bond formation, transport, and maturation of vacuolar carboxypeptidase Y. Moreover, cells bearing two other mutants with C-terminal shortened sequences but still containing one -CGHC- active site intact and 33 and 12% native isomerase activity, are no longer viable. So their conclusion was that catalysis of protein disulfides formation by PDI is not essential in yeast viability. PDI may therefore function directly in the protein folding pathway, acting, perhaps through its peptide-binding domain, absent in the two C-terminal shortened mutants, in a chaperone-like fashion.

Recently a mutant PDI with -SGHC- instead of -CGHC- at both active sites completely devoid of isomerase activity *in vitro* was found to increase the correct folding and secretion of human lysozyme co-expressed in yeast to the same extent as wide type PDI does [31] and this is claimed to be the first finding that PDI without isomerase activity accelerates protein folding *in vivo*.

It is known that PDI is identical with the essential β-subunit of the tetrameric prolyl-4-hydroxylase $\alpha_2\beta_2$ [32], and functions to prevent the misfolding and aggregation of the α-subunit so as to form the functional $\alpha_2\beta_2$ tetramer [33]. Vuori et al. [34] showed that a mutant β-subunit with a sequence of -SGHC- in both of the two active sites is devoid of isomerase activity, but still has the same function to associate with the α-subunit to form the fully active $\alpha_2\beta_2$ tetramer of prolyl-4-hydroxylase suggesting that some function other than the isomerase activity of PDI is involved in the assembly of the active hydroxylase tetramer.

Similarly, Wetterau et al. [35] showed that PDI is a component of the microsomal triglyceride transfer protein complex which consists of PDI and a 97 kD component. PDI is necessary to maintain the active structure of this microsomal protein, as the 97 kD component aggregates once dissociated from PDI [36]. Recently, the role of PDI in microsomal triglyceride transfer protein complex has been studied further by expressing the 97 kD subunit together with an inactive PDI in which both catalytic site sequences have been converted into -SGHC- and the results indicated that the disulfide isomerase activity of PDI is not required for its function of forming an active complex with the 97 kD subunit [37]. In both of the above cases, PDI, but not its isomerase activity, is essential for th assembly of the functional proteins although it differs from typical chaperones in that it is a stable component of the eventual functional structure.

THE PEPTIDE BINDING SITE OF PDI IS RESPONSIBLE FOR ITS CHAPERONE ACTIVITY

PDI is capable of binding peptides with low specificity [10, 11] and at least one peptide binding site in the PDI molecule distinct from the -CGHC- active sites has been identified [27], which was suggested to be responsible for its molecular chaperone-type function. Yeast bearing a mutant PDI shortened from the C-terminal, containing one -CGHC- active site but devoid of the putative peptide binding region midway between the active sites of native PDI can hardly survive [30].

Recently a mutant human PDI with its C-terminal 51 amino acid residues responsible for peptide binding deleted, abb'a', has been expressed in *E. coli* and showed neither peptide binding ability nor chaperone activity in assisting the refolding of denatured GAPDH, but kept most of its catalytic activities for reduction of insulin and isomerization of scrambled ribonuclease [38]. This provides a straightforward demonstration that the peptide binding site near the C-terminal of PDI is directly responsible for its chaperone activity. In this connection, thioredoxin, containing a similar -CGPC- active site but devoid of the peptide binding property [27] shows no effect on the refolding of GAPDH even at high concentrations [28].

The fact, that nonspecific peptides compete with the target protein for binding to PDI to prevent the PDI-assisted refolding and suppressing aggregation of the target proteins, confirms the role of the peptide binding site on its chaperone activity [28]. The bacte-

rial counterpart of eukaryotic PDI, DsbA, has a peptide binding site as shown in its high resolution 3D structure [39] and the DsbA-assisted refolding of GAPDH was likewise reduced with increasing concentrations of a peptide in the refolding buffer [40].

A pancreas-specific PDI with two thioredoxin-like active sites (-CGHC- and -CTHC-) was found to lack a sequence rich in acidic amino acids at the C-terminal functioning as a peptide binding domain, which accounted for the low enzyme activity compared to tissue-nonspecific PDI [41]. Also, the deletion of the N-terminal three amino acid residues of the peptide binding region of human PDI prevents the formation of active prolyl-4-hydroxylase tetrameter [37, 42].

BOTH *in vivo* AND *in vitro* PDI BEHAVES MORE THAN BEING AN ISOMERASE ONLY

PDI has been found to bind with non-native proteins and secretory proteins. Based on the ability of chaperone proteins to bind selectively unfolded proteins and to dissociate from them upon ATP hydrolysis, an affinity chromatography method has been developed by Nigam et al. [43] to isolate endoplasmic reticulum (ER) proteins with these properties. These proteins include some of the ER chaperones as well as members of the thioredoxin superfamily, i.e., PDI and Erp72, which appear to function as chaperones.

A complex of PDI with immunoglobulin was demonstrated by Roth and Pierce [44] using *in vivo* cross-linking suggesting that PDI transiently associates with newly synthesized proteins during the folding process in the ER. Otsu et al. [45] has directly characterized the association of PDI with misfolded mutant human lysozyme, but not with the native enzyme *in vivo* and related this to PDI's possible chaperone function, rather than to its isomerase activity. Similar results for PDI binding with non-native proteins have also been obtained by Persson and Petterson [46]. Chessler and Byers [47] found that PDI forms a stable complex with a mutated type I procollagen triplex in an abnormal conformation suggesting that PDI might play a role in its specific recognition, binding and therefore retention of improperly folded collagen molecules in the ER.

Like other "stress proteins" in ER, such as Bip and calreticulin, PDI was found to be induced by conditions generated by overproduction of secretory proteins or by the accumulation of unfolded or misfolded proteins within the ER [48]. Recently, both tissue-nonspecific PDI [49] and pancreas-specific PDI [50] were observed to be in transient contact with different secretory proteins, including proteins containing no cysteine residues during late stages of translocation. It was suggested that PDI may play a role in completion of cotranslational as well as posttranslational translocation and that this function of PDI may be related to its general chaperoning activity.

PDI catalyzes the assembly of the human chorionic gonadotropin α- and β-subunits both in intact cells and *in vitro* without being a component of the functional heterodimeric hormone molecule αβ [51]. It is interesting to note that the incompletely folded β-subunit is more assembly-competent than its fully disulfide bond form and bond preferentially by PDI.

Gunther et al. [52] reported that members of the mammalian PDI family, PDI or Erp72, are capable of replacing at least some of the critical functions of the Trgl protein essential for yeast viability and claimed that this gives the first hint for a possible thiol-specific chaperone function of the Trgl/Pdil protein *in vivo*.

Ostermeier et al. [53] considered the chaperone-like activity of PDI may play a role in the increased levels of BPTI co-expressed in *E. coli*. Nakamura et al. [54] considered that PDI catalyzed reductive cleavage of mixed disulfide through a chaperone-like function, i.e., interacts non-covalently with the protein to change its conformation necessary for both reduction and formation of disulfide. They also found the mutant PDI containing two -SGHC- instead of -CGHC- in the active sites did not assist in the dissociation of other glutathione derivatives, however retain its normal recognition ability and chaperone-like function.

In a recent review on assisted protein folding it has been stated that PDI and PPI are usually considered as ER chaperones [55]. On the other hand, Raines claimed that the primary role of PDI is catalysis by its thioredoxin-like domain and chaperone function is less relevant and not essential [56].

THE FOLDASE ACTIVITY OF PDI CONSISTS OF BOTH ITS ISOMERASE AND CHAPERONE ACTIVITIES

Yao et al. reported recently [57] that PDI assists the oxidative refolding and reactivation of denatured and reduced acidic phospholipase A_2 ($APLA_2$), a small snake venom protein containing 7 disulfide bonds, and 90% of the native PDI present in the refolding buffer can be fully replaced by a modified PDI (mPDI) with only chaperone activity but no isomerase activity [28], indicating that the *in vitro* action of PDI as a foldase consists of both isomerase and chaperone activities. mPDI alone is completely inactive in promoting the refolding of $APLA_2$ and abb'a' is only weakly active, but the simultaneous presence of both in the refolding buffer does show a co-operative action to promote the reactivation of $APLA_2$, although a much higher amount of abb'a' is required for the maximal reactivation compared to native PDI, suggesting a higher efficiency of PDI with both functions in the same molecule as compared to the

combined action of two molecules in abb'a' for the isomerase and mPDI for the chaperone [38].

The above findings are reminiscent of a chaperone in *E. coli*, trigger factor, which contains a central domain belonging to the FK506-binding protein family and is a ribosome-bound PPI [58-60]. The trigger factor also has a peptide binding site well separated from the isomerase active site and responsible for its high efficiency as a catalyst of protein folding with peptidyl-proline isomerization as the rate limiting factor [61]. The trigger factor shows a much more effective folding-catalyzing ability for big proteins than all three kinds of small PPIs *via* its chaperone-like function. While the small PPIs catalyze prolyl isomerization much better in short unstructured polypeptides than in the refolding of proteins, because in addition to prolyl isomerization, protein folding involves very complicated folding events which chaperone can handle. A excised central fragment corresponding to the FK506-binding protein from trigger factor molecule remains fully active as a prolyl isomerase with a short peptide as a substrate, but its activity is reduced about 1000-fold in assisting protein folding.

The fact that PDI and the trigger factor both consist of two parts, the catalytic and the peptide binding parts, seems to imply an evolutionary consequence for more efficient assistance of protein folding. Compared to thioredoxin, PDI acquired an additional -CXYC- containing domain, as well as a domain for peptide binding during evolution so as to function as a foldase with both isomerase and chaperone activities, while thioredoxin, showing neither peptide binding ability nor chaperone activity, has a much lower isomerase activity than PDI. Such is also the case for the trigger factor as compared with the small PPIs.

A close relation of both isomerase and chaperone activities has also been found in a well-established chaperone DnaJ in *E. coli*, which possesses the -CXYC- sequence and can catalyze protein disulfide formation, reduction, and isomerization like PDI [62].

PROPOSED SCHEME OF PDI ACTION ON PROTEIN FOLDING

PDI, like other chaperones, recognizes and binds with non-native structure of unfolded or partially folded intermediates of nascent peptides or denatured proteins during folding through its peptide binding site(s) thus preventing incorrect association and aggregation of its target substrate. Unlike the ATP-dependent chaperones, such as the GroE system, but like some other chaperones, such as Hsp90 [63, 64], the binding of PDI with its substrates might be transient and the dissociation of the complex does not depend on the presence of ATP, which is usually necessary for the release and further refolding of the target proteins assisted by most chaperones, such as Hsp60 and Hsp70 [65, 66]. In such a way PDI prevents non-productive interactions of nascent peptide chains leading to aggregation and possible subsequent degradation *in vivo*. The transient association of PDI with target proteins promotes their correct folding to native-like conformation so as to bring the corresponding sulphydryl groups into proximity to be joined by oxidative formation of native disulfides and this latter reaction is the generally recognized function of PDI. Therefore, it is not surprising that PDI is capable of nonspecific peptide binding which is the molecular basis of PDI being a chaperone. The chaperone and the isomerase activities are in a way independent from each other but function in collaboration, possibly at consecutive stages of the folding process of the target protein. At an early stage of the folding process, PDI functions mainly as a chaperone to block the incorrect association of partially folded chains prone to aggregation. It primarily functions as an isomerase at a subsequent stage when the peptide chain has already folded to a certain extent for the oxidative linkage of the paired sulphydryl groups and/or isomerization of incorrectly linked disulfide.

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